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Site-Specific Inhibition of RNA Polymerase II Preinitiation Complex Assembly by Human Cytomegalovirus IE86 Protein†

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The human cytomegalovirus major immediate-early gene encodes several protein isoforms which autoregulate the major immediate-early promoter (MIEP). One of these isoforms, the IE86 protein (UL122, IE2), is a DNA-binding protein that represses the MIEP through its cognate recognition sequence (designated the *cis* repression signal [*crs*]) located between the TATA box and the initiation site of transcription. Purified recombinant IE86 protein was shown to repress MIEP transcription in vitro, in a *cis*-acting mediated pathway, with nuclear extracts from HeLa S3, U373-MG, and primary human foreskin fibroblast cells. Repression of the MIEP by IE86 was shown by two criteria to be dependent on the direct interaction of IE86 with the *crs* element. Core promoter constructs containing essentially the MIEP TATA box and *crs* element were also specifically repressed by IE86 but not by a mutant IE86 protein, indicating the general transcription machinery as the target for IE86 repression. Kinetic and template commitment experiments demonstrated that IE86 affects preinitiation complex formation but not the rate of reinitiation. Sarkosyl inhibition experiments further revealed that IE86 was unable to effect repression by either disassembling or preventing the elongation of a preexisting transcription complex. Further, the ability of IE86 to interact with the DNA-binding subunit of TFIID was shown not to be required for repression. These functional protein-DNA and protein-protein interaction experiments demonstrate that IE86 specifically interferes with the assembly of RNA polymerase II preinitiation complexes. The biological significance of these results and the precise mechanism by which IE86 represses transcription are discussed.

The immediate-early (IE) genes of human cytomegalovirus (HCMV) encode transcriptional regulatory proteins which, together with host-encoded transcription factors, temporally regulate the developmental expression of the viral genome. We are interested in understanding the role of viral and cellular proteins in coordinating RNA polymerase II (RNAPII) activity associated with HCMV gene regulation.

The HCMV IE86 protein (also referred to as UL122 or IE-2 80- or 82-kDa protein) is translated from an mRNA derived from region 1 and 2 genomic sequences of the major IE (MIE) gene, which is transcriptionally regulated by the MIE promoter (MIEP) (20, 37, 43, 44, 46). The IE86 protein is an activator of a variety of promoters including those of the HCMV early genes, as well as heterologous viral and cellular promoters (2, 3a, 5–8, 13, 17, 19, 24, 30, 35, 37–40, 42, 47–49). The precise mechanism(s) by which this protein activates transcription is not clearly understood. However, a recent study by Klucher et al. (24) implicates antirepression of transcription by histone H1 as one possible mechanism underlying IE86 activation. Another possibility for the promiscuous action of IE86 might involve the direct interaction of IE86 with general transcription factors required for establishing RNAPII initiation complexes. In support of this suggestion, IE86 has been shown to directly interact with the TATA box-binding protein (TBP) in the absence of DNA (14) and with promoter-bound TBP (22). Transcription initiation from eukaryotic protein-encoding genes is a multistep process that requires RNAPII and as many as seven general transcription factors (reviewed in reference 50). The TBP subunit of the general transcription factor TFIID mediates the recognition of the TATA sequence element and represents the first step in the formation of a preinitiation complex (50). Significantly, IE86 has been shown to stimulate the binding of TBP to promoter DNA, thus affecting a critical rate-limiting step in the assembly of an initiation complex (22). Furthermore, the TBP-contacting domain of IE86 responsible for mediating the interaction of IE86 with promoter-bound TBP partially overlaps with the N-terminal activation region of the protein (22).

In addition to the ability of this protein to stimulate transcription, IE86 has been shown to negatively autoregulate the MIEP (1, 4, 16, 28, 36, 38, 42). Negative regulation of the MIEP by IE86 is dependent on a sequence element termed the *cis* repression signal (*crs*) located between the TATA box and the cap site (4, 28, 36). IE86 can bind directly to the *crs* element (25) via a DNA binding domain located in the C terminus of the protein (21, 29). Evidence that the binding of IE86 to the *crs* element is responsible for repression has recently been provided by the ability of an IE86 maltose-binding fusion protein to repress transcription in vitro (29). Since the *crs* element is positioned immediately proximal to the TATA box, it is possible that IE86 might affect TBP binding to the promoter site. Recently, we have shown that TBP and IE86 can simultaneously bind to their cognate recognition sites on the MIEP (21), suggesting that IE86 might influence subsequent assembly steps in the preinitiation process. These steps involve TBP's association via protein-protein interactions with the general transcription factors TFIIA (DA complex) and TFIIB (DB complex) or with both TFIIA and TFIIB (DAB complex). The DAB or DB complex acts as the preinitiation complex for

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the entry of RNAPII, mediated by TFIIF, into the transcription cycle (reviewed in reference 50). Recently, a number of factors that interact with TBP have been shown to functionally repress basal transcription (18, 31, 32). Therefore, the interaction of IE86 with TBP may also be essential for the negative regulation of the MIEP by IE86. Alternatively, repression of MIEP activity by IE86 may proceed by a mechanism independent of this interaction. For instance, IE86 may effect inhibition of transcription post-preinitiation complex formation by either directly disassembling a preexisting complex or hindering the processivity of an elongating RNAPII complex.

Autorepression of the MIEP by IE86 is critical for MIEP activity which may ultimately affect the permissiveness of the virus within the cell. Therefore, knowledge of the step(s) in the assembly of transcription complexes at which IE86 effects inhibition will be fundamental to the understanding of this process. In this study, we have attempted to address these issues by defining the molecular requirements by which IE86 autorepresses the MIEP in an in vitro transcription system. Evidence is presented to indicate at which step in the transcription process IE86 exerts its negative effects.

MATERIALS AND METHODS

Recombinant plasmids. The DNA templates pMIEP (−1145/+112)CAT, pMIEP(−65/+7)CAT, and pMIEP(mcrs)CAT used in the in vitro transcription assays have been described previously (1, 12). The DNA template pRR56/5 was a kind gift from B. Fleckenstein. The construction of the His-6-tagged IE86 expression clone (p86-6His) and the generation of IE86 mutant expression clones for IE86mZn, IE86ΔN6, IE86ΔMS, IE86ΔC2, IE86ΔN1ΔC2, and IE86ΔN6ΔC2 are described elsewhere (21, 22).

Purification of recombinant proteins. *Escherichia coli* harboring each of the expression plasmids was grown to an optical density at 550 nm of 0.7 to 0.8 prior to induction with 200 μg of isopropyl-β-D-thiogalactopyranoside (IPTG) per ml. Cells were harvested after 90 to 120 min of induction and stored at −70°C. Cells were thawed and then lysed in buffer containing 50 mM sodium phosphate (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 1% Tween 20, 1 M NaCl, and 1 mg of lysozyme per ml for 20 min prior to sonication. Following centrifugation at 16,000 rpm in a Sorvall SS34 rotor, the cleared lysate was subjected to Ni^{2+} chelate chromatography (Qiagen, Chatsworth, Calif.) (26) over a column equilibrated in buffer containing 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, and 10% glycerol. After being washed with this buffer, the column was washed in a similar buffer at pH 6.0. A final wash was performed with the latter buffer containing 75 mM imidazole prior to elution in the same buffer containing 500 mM imidazole. Fractions containing each of the proteins were pooled and dialyzed against 50 mM sodium phosphate (pH 7.8)–250 mM NaCl–1 mM β-mercaptoethanol–30% glycerol. Analysis of the protein fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the IE86 and IE86 mutant proteins were approximately 90% pure.

Nuclear extract preparation and in vitro transcription assays. The nuclear extracts derived from HeLa S3, U373-MG, and primary human foreskin fibroblast (HFF) cells were prepared from exponentially growing cells as described previously (9), with the exception that all buffers contained 10 mM (each) leupeptin, pepstatin, and aprotinin as well as 0.5 mM phenylmethylsulfonyl fluoride. Protein concentration was determined by the Bradford method (3).

The transcription reaction conditions (in 25 μl) were as described previously (11). Routinely, poly(U) polymerase activity present in the extracts was used as an internal control to account for variability during the workup of the RNA samples. Optimal DNA concentrations were determined for the constructs pMIEP(−1145/+112)CAT and pMIEP(mcrs)CAT in the different cell types. These corresponded to 10 and 20 μg/ml for pMIEP(−1145/+112)CAT and pMIEP(mcrs)CAT in the HeLa nuclear cell extract, respectively. For U373-MG transcription reactions, 15 μg of the wild-type and mutant templates per ml was used. The pMIEP(−1145/+112)CAT and pMIEP(mcrs)CAT templates were linearized with *Eco*RI-*Hin*dIII and *Pvu*II, respectively, prior to use. The templates pMIEP(−65/+7)CAT and pRR56/5 were each linearized with *Eco*RI and *Hin*dIII and used in the transcription reactions at a concentration of 25 μg/ml. In the template commitment experiments, pMIEP(−1145/+112)CAT was resected with *Eco*RI and *Hin*dIII for template 1 and *Pvu*II for template 2, respectively. The final concentration of these templates used in the template commitment assays was 3 μg/ml. The Sarkosyl addition experiments designed to dissociate the initiation and elongation steps of transcription were based on the studies of Hawley and Roeder (15).

RESULTS

In vitro repression of transcription by IE86. To examine whether the ability of IE86 to bind the *crs* element is a prerequisite to the mechanism by which IE86 mediates repression of MIEP transcription, in vitro transcription from the MIEP with nuclear extracts prepared from both nonpermissive and permissive cells was analyzed in the absence or presence of increasing amounts of purified recombinant IE86 protein. In nonpermissive HeLa cell nuclear extract, 75 nM IE86 was observed to abolish transcription from a template containing the complete MIEP control sequence, pMIEP(−1145/+112)CAT (Fig. 1A, lane 3). More importantly, repression of this construct was also observed in the presence of IE86 in an in vitro transcription assay using extracts prepared from a permissive cell line (U373-MG cells) and permissive primary HFF cells (Fig. 1B and C, lanes 2 to 5). A concentration of IE86 similar (corresponding to an equivalent number of molecules of IE86 per DNA template) to that observed for the HeLa transcription was also required in reaction mixtures from the permissive cells to shut off transcription. By contrast, no significant repression of transcription from an MIEP template [pMIEP(mcrs)CAT], in which clustered point mutations were present in the *crs* element, was observed with either the HeLa, U373-MG, or HFF nuclear extracts (Fig. 1A to C, lanes 7 to 10 compared with lane 6). The pMIEP(mcrs)CAT construct has been previously shown to be nonresponsive to IE86 repression in vivo (1). Although marginal repression by IE86 at high concentrations (>150 nM) was observed with the pMIEP (mcrs)CAT template in HFF nuclear extracts (Fig. 1C, lanes 9 and 10), lower concentrations of IE86 (75 nM), which abolished transcription with the wild-type template, were unable to effect inhibition with pMIEP(mcrs)CAT (Fig. 1C, compare lane 3 with lane 8). These results also indicate that the inhibition by recombinant IE86 is not due to a trivial reason, such as the presence of a contaminating RNase activity.

To test directly whether the binding of IE86 to the *crs* element is required to repress transcription from the MIEP, the effect of a mutant form of IE86 that is incapable of binding the *crs* element was investigated. The mutant of IE86 (IE86mZn) used in these experiments contains a 2-amino-acid (aa) substitution mutation of the two cysteine residues (C-428 and C-434 converted to serine residues) located within the putative zinc finger domain and has been previously shown to

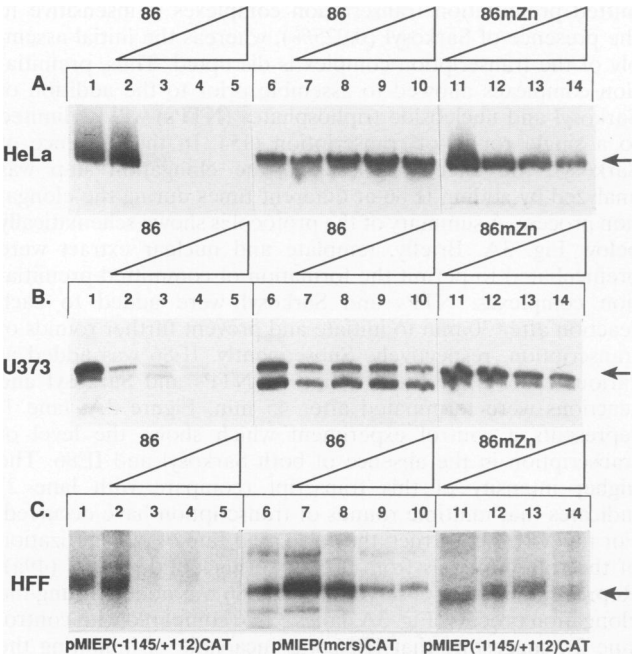

FIG. 1. In vitro repression of MIEP transcription by IE86 with HeLa (A), U373-MG (B), and HFF (C) nuclear extracts. Each of the nuclear extracts was tested, with pMIEP(−1145/+112)CAT (lanes 1 to 5 and 11 to 14) and pMIEP(mcrs)CAT (lanes 6 to 10), in the absence (lanes 1 and 6) or presence of increasing amounts of IE86 wild-type protein (86) (lanes 2 and 7, 38 nM; lanes 3 and 8, 75 nM; lanes 4 and 9, 150 nM; and lanes 5 and 10, 190 nM) or IE86 zinc finger mutant protein (86mZn) (lanes 11, 80 nM; lanes 12, 150 nM; lanes 13, 320 nM; and lanes 14, 400 nM). Nuclear extracts, templates, and IE86 proteins as indicated were preincubated for 30 min prior to the addition of NTPs and the initiation of transcription. Reactions were stopped 45 min after addition of NTPs. Arrows indicate specific transcript. Note that in U373-MG transcription reactions a prematurely terminated transcript [most notable for the pMIEP(mcrs)CAT template] is also observed.

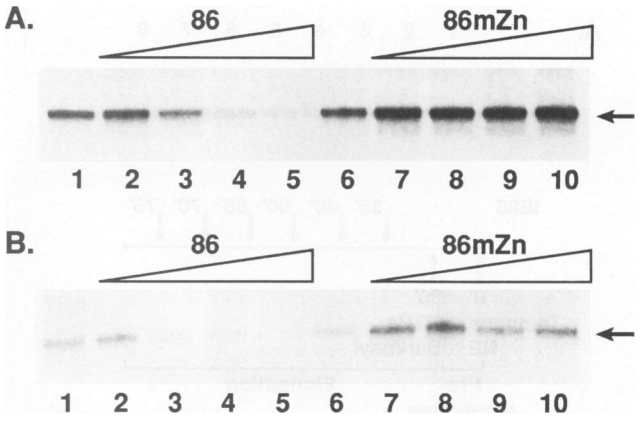

FIG. 2. IE86 repression of the MIEP core promoter. (A) In vitro transcription (HeLa nuclear extract) from pRR56/5 template in the absence of added IE86 protein (lanes 1 and 6) and with increasing concentrations of either wild-type IE86 (lanes 2 to 5) or mutant IE86mZn (lanes 7 to 10) corresponding to 38 nM (lanes 2 and 7), 75 nM (lanes 3 and 8), 150 nM (lanes 4 and 9), and 190 nM (lanes 5 and 10) of recombinant protein. (B) In vitro repression of core-promoter template pMIEP(−65/+7)CAT. Lanes are as described for panel A. Arrows, specific transcript.

be phenotypically defective in binding the *crs* element (21, 29). In both the permissive (U373-MG and HFF) and the nonpermissive (HeLa) cell extracts, no significant repression of transcription was observed even in the presence of 320 nM IE86mZn (Fig. 1A to C, lanes 13). However, a marginal decrease was observed with 400 nM IE86mZn (Fig. 1A to C, lanes 14), but this most likely reflects a nonspecific effect at this extremely high protein concentration. These experiments in combination with previous data (21, 25, 29) demonstrate that the direct binding of IE86 to the *crs* element is a requirement for mediation of transcriptional repression. The observation that IE86 is capable of repressing transcription in a variety of different cell extracts indicates that its effects are not cell type dependent, thus implicating the general transcription machinery as the target for IE86 action.

IE86 repression is independent of the upstream enhancer and downstream transcriptional control domains of the MIEP. To examine directly whether IE86 repression of the MIEP involves stimulatory sequences from the enhancer domain, a DNA template (pRR56/5) in which sequences upstream of nucleotide position −65 have been deleted but which contains downstream (to nucleotide position +54) control elements was assayed for in vitro repression by IE86. Note that the ability of the upstream enhancer elements and the downstream control elements to stimulate in vitro transcription from the MIEP has been previously shown (11, 12). In the presence of increasing concentrations of IE86, transcription from the pRR56/5 template was shown to be repressed by IE86 in a dose-dependent manner (Fig. 2A, lanes 2 to 5). A complete inhibition of transcription was observed with a concentration of IE86 between 75 and 150 nM. To show that the repressive effect was specific, a similar titration experiment was performed with increasing concentrations of an IE86 mutant protein (IE86mZn). In this experiment (Fig. 2A, lanes 6 to 10), repression of transcription was not observed in the presence of 190 nM IE86mZn protein. These experiments indicate that the upstream enhancer domain is not required for repression by IE86.

To examine further whether the downstream activation domain, in the absence of the enhancer domain, could be a requirement for IE86 repression, the DNA template pMIEP(−65/+7)CAT, which encompasses MIEP sequences between nucleotide positions −65 and +7, was tested in the in vitro repression assay. This construct essentially contains the TATA and *crs* element but lacks the upstream enhancer and downstream transcriptional control domains. Similar to the pRR56/5 template, pMIEP(−65/+7)CAT was found to be repressed by IE86 in a dose-dependent manner (Fig. 2B, lanes 2 to 5), in which complete inhibition of transcription was observed with a 75 nM concentration of IE86 (Fig. 2B, lane 3). Furthermore, repression was not observed with this template in the presence of increasing concentrations of the IE86mZn protein, indicating the specificity of the repression observed with the IE86 protein (Fig. 2B, lanes 6 to 10). These experiments indicate that IE86 can repress the core promoter of the MIEP which is dependent on the assembly of the general transcription factors and RNAPII.

IE86 acts on RNAPII preinitiation complex formation but not on reinitiation or elongation steps. To elucidate the possible mechanism by which IE86 interacts with the general transcription machinery, a series of kinetic and Sarkosyl inhibition experiments were designed. In the first experiment (Fig. 3A), we examined whether IE86 could interfere with the elongation step of a committed complex. Elongation of com-

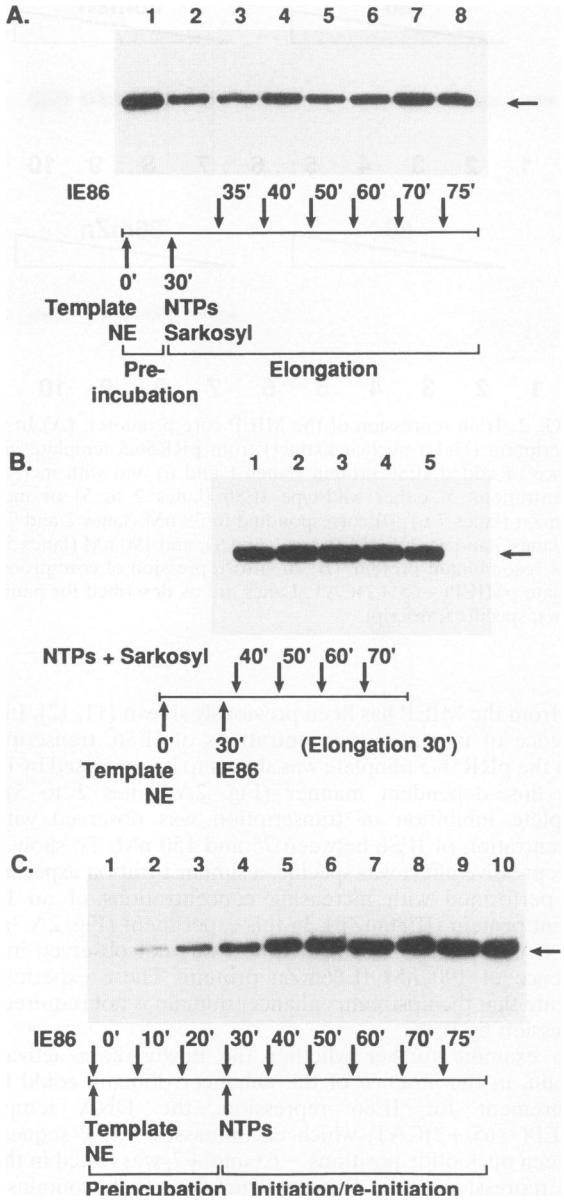

FIG. 3. IE86 protein acts on RNAPII preinitiation complex formation but not on reinitiation or elongation steps. (A) pMIEP(−1145/+112)CAT resected at the *Eco*RI site (generating runoff transcripts of 365 nucleotides) was preincubated with HeLa cell nuclear extract for 30 min. Transcriptions were started by adding NTPs and Sarkosyl at a final concentration of 0.025% (wt/vol) (lanes 2 to 8). IE86 protein (190 nM final concentration) was added at the times indicated (lanes 3 to 8). Reactions were stopped 45 min after addition of NTPs. Transcription without Sarkosyl and IE86 protein (lane 1) was taken as the control for multiple rounds of transcription. (B) Preincubation was the same as described above. IE86 protein was added at 30 min (lanes 1 to 4). Transcriptions were started by adding NTPs and Sarkosyl at the times indicated. For the control (lane 5) without IE86, transcription was started by adding NTPs and Sarkosyl at 30 min. Each reaction was stopped after 30 min of elongation time. (C) pMIEP(−1145/+112)CAT and HeLa nuclear extracts were preincubated for 30 min, and then transcription was started by adding NTPs. IE86 protein (190 nM final concentration) was added as indicated (lanes 1 to 9). The reaction was stopped 45 min after the addition of NTPs. Transcription repressed by IE86 protein was compared with transcription without IE86 protein (lane 10). NE, nuclear extract.

mitted preinitiation transcription complexes is insensitive to the presence of Sarkosyl (0.025%), whereas the initial assembly of the transcription complex is disrupted. Thus, preinitiation complexes allowed to assemble prior to the addition of Sarkosyl and nucleoside triphosphates (NTPs) will be limited to a single round of transcription (15). In the presence of Sarkosyl, the effect of IE86 on the elongation step was analyzed by adding IE86 at different times during the elongation process. A summary of the protocol is shown schematically below Fig. 3A. Briefly, template and nuclear extract were preincubated to permit the formation of committed preinitiation complexes. NTPs and Sarkosyl were added to each reaction after 30 min to initiate and prevent further rounds of transcription, respectively. Subsequently, IE86 was added at various times after the addition of NTPs and Sarkosyl and reactions were terminated after 45 min. Figure 3A, lane 1, represents a control experiment which shows the level of transcription in the absence of both Sarkosyl and IE86. The higher intensity of this transcript (compare with lane 2) indicates that multiple rounds of transcription have occurred. For the MIEP construct, the calculated rate of polymerization of the transcript was from 0.2 to 0.6 nucleotides per s (49a). Repression was not observed when IE86 was added during the elongation process (Fig. 3A, lanes 3 to 6 compared with control lane 2), suggesting that IE86 was incapable of inhibiting the elongation of RNAPII complexes.

Competent preinitiation complexes (formed in the absence of NTPs) are only those complexes which are capable of rapidly initiating transcription upon addition of NTPs to the reaction. Therefore, in the second experiment (Fig. 3B), the ability of IE86 to disassemble preformed preinitiation complexes was addressed. Template DNA was preincubated with nuclear extract for 30 min to permit assembly of the preinitiation complexes. IE86 was then added, to allow for the interaction of IE86 with committed preinitiation complexes at designated times prior to the initiation of a single round of transcription (Fig. 3B). The elongation time after the addition of NTPs and Sarkosyl was 30 min for each reaction. The amount of competent preinitiation complexes was assessed by the quantity of transcripts on the gel. The protocol followed is shown schematically below Fig. 3B. A decrease in the quantity of runoff transcripts with increasing exposure to IE86 would be observed if IE86 disassembled competent preinitiation complexes. However, IE86 was not capable of repressing transcription from the preformed preinitiation complexes (Fig. 3B, lanes 1 to 4 compared with control lane 5), suggesting that this step in the transcription process is not the critical step for IE86-mediated repression.

In the third experiment, we investigated whether IE86 can inhibit the formation of preinitiation complexes. As shown in the protocol summary (Fig. 3C), IE86 was added to the transcription reaction at different times during formation of an RNAPII preinitiation complex as well as during the initiation/reinitiation stages (post-addition of NTPs). The preincubation and the initiation/reinitiation times indicated in Fig. 3C refer to time allowed for preinitiation complex assembly and productive transcription from committed complexes, respectively. The template pMIEP(−1145/+112)CAT DNA was incubated with the nuclear extract for 30 min at 25°C, during which IE86 was added at 0, 10, 20, and 30 min. At 30 min, NTPs were added, and subsequently, IE86 was included in the reactions at 40, 50, 60, 70, and 75 min. Each reaction was stopped 45 min after NTP addition, and the amount of transcript produced in each reaction was monitored by gel electrophoresis.

IE86 disruption of preinitiation complex formation would be indicated by a decrease in transcription during the preincuba-

tion period only. However, if IE86 repressed transcription subsequent to preinitiation complex formation, its effect on transcription would be predicted to occur both before and after addition of NTPs. Repression of transcription by IE86 was effective within the first 10 min during the formation of the preinitiation transcription complex (Fig. 3C, lanes 1 and 2 compared with lane 10). Inhibition of transcription by IE86 gradually decreased between 10 and 30 min (Fig. 3C, lanes 3 and 4). Upon initiation of a committed preinitiation complex, the amount of transcript produced remained constant with no observed repression (Fig. 3C, lanes 5 to 9). This experiment demonstrated that IE86 affected a relatively early step in the formation of an RNAPII preinitiation complex (Fig. 3C). Since the conditions of this experiment (Fig. 3C) were not restricted to a single round of transcription, components of the preinitiation complex still present at the promoter site would enable reinitiation of transcription after engagement of RNAPII in the first round. If IE86 were able to repress transcription at this step, an inhibitory effect of IE86 after addition of NTPs would have been observed. However, IE86 failed to repress transcription under these conditions, in which initiation and reinitiation complexes were allowed to form (that is, post-NTP addition) (Fig. 3C, lanes 5 to 9), suggesting that IE86 cannot affect the reinitiation step in transcription. In summary, these three experiments (Fig. 3A to C) are consistent with the conclusion that IE86 targets specifically the formation of a preinitiation complex.

Committed preinitiation complexes are refractory to repression by IE86. In order to independently confirm the role of IE86 in the inhibition of preinitiation complex assembly, we performed template commitment experiments in which initiation complexes were preformed on one experimental template and then challenged with a second virgin template while concomitantly initiating transcription by the addition of NTPs. Subsequently, IE86 was added to the reaction at various times after initiation, and the relative level of transcription was measured from both templates. The pMIEP(−1145/+112)CAT construct was used to generate both template 1 and template 2 by truncating the DNA at different sites downstream of the cap site so that the respective runoff transcripts could be readily distinguished by gel electrophoresis and consequently monitored simultaneously in a single transcription reaction.

First, we established conditions that permitted approximately equivalent levels of transcription from simultaneously added template 1 and 2 DNAs in the absence of IE86 (Fig. 4, lane 1). The results of this experiment demonstrate that there are sufficient levels of transcription factors in the reaction for assembly and elongation of transcription complexes on both templates 1 and 2. With these conditions, the ability of IE86 to simultaneously inhibit initiation complexes on both templates 1 and 2 was examined. In a manner parallel to that of the previous kinetic experiments (Fig. 3C, lanes 1 to 4), IE86 was incubated with the templates (1 and 2) and nuclear extract at 0, 5, 10, 20, and 30 min prior to the addition of NTPs. The reaction was stopped 30 min after NTP addition, and the amount of transcript produced in each reaction was monitored by gel electrophoresis. A schematic summary of this protocol is shown in Fig. 4, part I. The results of this experiment (Fig. 4, lanes 2 to 6 compared with control lane 1) demonstrate the ability of IE86 to inhibit initiation complexes on both templates 1 and 2 with maximal inhibition occurring within the first 10 min of the formation of the preinitiation complexes (lanes 2 to 4). Those reactions in which IE86 was added within 10 to 30 min of preinitiation complex formation (Fig. 4, lanes 4 to 6) varied slightly in the extent of repression observed for template 1 relative to template 2. The reason for this observation is

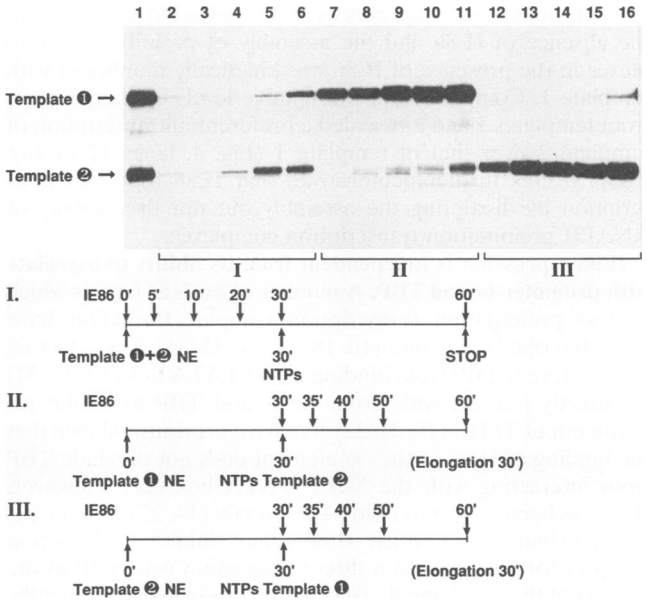

FIG. 4. Template commitment assays. pMIEP(−1145/+115)CAT was resected by *Eco*RI and *Hin*dIII (template 1) and *Pvu*II (template 2), respectively. Templates 1 and 2 generated predicted transcripts of 365 and 265 nucleotides (indicated by arrows), respectively (lane 1). (I) Both templates (each at a final concentration of 3 μg/ml) and HeLa nuclear extract (NE) were preincubated for 30 min. IE86 protein was added (190 nM final concentration) at the times indicated (lanes 2 to 6). (II) Template 1 was first preincubated with nuclear extract for 30 min, and then template 2 and NTPs were added to start transcription. IE86 protein was added as indicated (lanes 7 to 11). (III) The experimental procedure is exactly the same as for part II except that template 2 was first preincubated with nuclear extract (lanes 12 to 16). All reactions were stopped after 30 min of elongation time (after IE86 addition).

unclear but might indicate different rates of assembly of initiation complexes on the templates, different rates of processivity of RNAPII complexes, or different rates of sequestration of IE86. Nevertheless, these experiments show that there are sufficient amounts of IE86 present in the reaction to inhibit transcription complex formation on both templates.

To test the template commitment and repression of the initiation complexes by IE86, the operations outlined schematically in Fig. 4, parts II and III, were performed. If a competent preformed complex survived interference by IE86 on template 1 and IE86 inhibited the preinitiation complex assembly on template 2, there would be preferential transcription from template 1 relative to template 2. In the experiment whose results are shown in Fig. 4, part II, transcription complexes were allowed to assemble on template 1 in the absence of IE86. After 30 min, template 2 and NTPs were added and were followed by the addition of IE86 to the reaction at 30, 35, 40, 50, and 60 min. The reaction was stopped 30 min after IE86 addition, and the amount of transcript produced in each reaction was monitored by gel electrophoresis. Similar levels of transcription from templates 1 and 2 were observed in reactions which did not include IE86, indicating that transcription factors were not limiting (data not shown). As predicted, the preformed preinitiation complexes on template 1 were resistant to inhibition by IE86 while preinitiation complex formation on template 2 was sensitive to repression by IE86 (Fig. 4, lanes 7 to 11).

In the reciprocal template commitment experiment (Fig. 4,

part III), initiation complexes were committed to template 2 in the absence of IE86 and the assembly of preinitiation complexes in the presence of IE86 was kinetically monitored with template 1. Comparison of the relative levels of transcription from templates 1 and 2 revealed a preferential transcription of template 2 over that of template 1 (Fig. 4, lanes 12 to 16). These results further demonstrate that IE86 represses transcription by disrupting the assembly but not disassembly of RNAPII preinitiation transcription complexes.

IE86 repression is independent from its ability to associate with promoter-bound TBP. A number of nuclear factors which repress preinitiation transcription complex formation have been described elsewhere (10, 18, 23, 31, 32, 34). These factors either prevent TBP from binding to the TATA box (10, 23, 34) or directly interact with promoter-bound TBP to inhibit recruitment of TFIIB (18, 31, 32). We have previously shown that the binding of IE86 to the *crs* element does not preclude TBP from interacting with the MIEP TATA box (21). However, IE86 has been shown to bind TBP directly (14, 22). Therefore, one mechanism by which IE86 might inhibit preinitiation complex formation is via a direct association with TBP in the context of the *crs* element. To examine this hypothesis directly, mutant forms of IE86 compromised in their ability to bind either promoter-bound TBP or the *crs* element or both were assayed for their ability to repress transcription in vitro. The IE86 mutants used in these experiments are represented schematically in Fig. 5B. Briefly, IE86ΔN6 encodes an N-terminal deletion of the first 153 aa residues of IE86 while IE86ΔMS encodes an internal deletion of 13 aa residues (positions 141 to 153 of IE86); both of these mutants retain the ability to interact with the *crs* element but are incapable of binding promoter-bound TBP (21, 22). In contrast, IE86ΔC2, IE86ΔN1ΔC2, and IE86ΔN6ΔC2 are truncated at aa position 541 of IE86 and have lost their ability to bind the *crs* element (21). In addition, IE86ΔN1ΔC2 and IE86ΔN6ΔC2 have N-terminal truncations at aa positions 48 and 154, respectively. While IE86ΔN1ΔC2 retains the ability to interact with promoter-bound TBP, IE86ΔN6ΔC2 is defective for this TBP interaction (22). The template pMIEP(−1145/+112)CAT was preincubated for 10 min with increasing concentrations of the purified IE86 mutant proteins; preincubation was followed by the addition of nuclear extract and NTPs. The reaction was stopped 45 min after NTP addition, and the amount of transcript produced in each reaction was monitored by gel electrophoresis.

The results of the experiment shown in Fig. 5A demonstrate that IE86ΔN6 (lanes 5 to 7) and IE86ΔMS (lanes 8 to 10), but not IE86ΔC2 (lanes 11 to 13), IE86ΔN1ΔC2 (lanes 14 to 16), and IE86ΔN6ΔC2 (lanes 17 to 19), were capable of repressing the MIEP in a dose-dependent manner. However, the extent to which IE86ΔN6 and IE86ΔMS repress transcription is slightly less than that of the wild-type protein. Since IE86ΔN6 and IE86ΔMS can bind the *crs* element but are incapable of interacting with promoter-bound TBP, we suggest that the mechanism by which IE86 represses preinitiation complex formation is not entirely dependent on its ability to interact with promoter-bound TBP, in the context of the *crs* element. By contrast, those mutants that lack the ability to bind the *crs* element (IE86ΔC2, IE86ΔN1ΔC2, and IE86ΔN6ΔC2, including IE86mZn [Fig. 1, lanes 11 to 14] and IE55 [an isoform variant of IE86] [data not shown]) were ineffectual in repressing transcription from the MIEP, further emphasizing that the site-specific interaction of IE86 with the *crs* element is critical for the mechanism underlying IE86 repression.

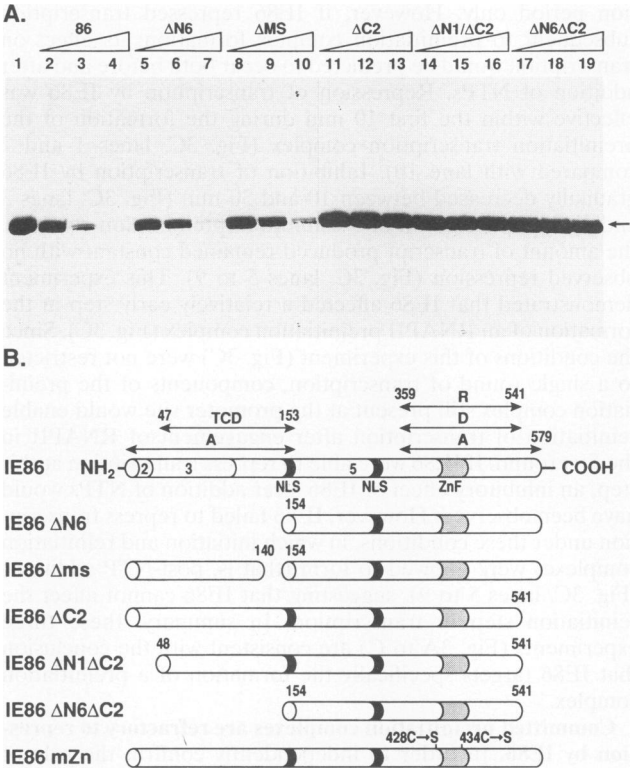

FIG. 5. IE86 repression is independent from its ability to contact promoter-bound TBP. (A) Transcription assays were performed as described in the legend to Fig. 2 with IE86 wild type (lanes 2 to 4), IE86ΔN6 (lanes 5 to 7), IE86ΔMS (lanes 8 to 10), IE86ΔC2 (lanes 11 to 13), IE86ΔN1ΔC2 (lanes 14 to 16), and IE86ΔN6ΔC2 (lanes 17 to 19). Concentrations of IE86 and mutant forms used in the reactions were 38 nM for lanes 2, 5, 8, 11, 14, and 17; 75 nM for lanes 3, 6, 9, 12, 15, and 18; and 190 nM for lanes 4, 7, 10, 13, 16, and 19. The effects of different IE86 proteins on transcription were compared with the control (lane 1). Note that these mutant proteins bound either DNA or TBP with efficiencies comparable to that of the wild-type IE86 protein. (B) Diagram of IE86 wild-type protein and different mutated IE86 proteins used in the transcription assays. Numbers refer to amino acid positions, except for the numbers shown in the rod, which designate amino acids encoded by exons 2, 3, and 5 of the MIE gene. Abbreviations: NLS, nuclear localization sequence; ZnF, putative zinc finger domain; TCD, TBP-contacting domain; D, DNA binding domain; A, activation domain; R, autorepression domain.

DISCUSSION

As part of our efforts to understand and elucidate the role of cellular and viral transcription factors in coordinating RNAPII activity associated with HCMV promoters, we have undertaken an in vitro analysis of repression by the IE86 protein, one of the predominant products expressed from the MIE gene during HCMV infection.

IE86 acts at an early stage of initiation complex assembly. Consistent with previous studies (1, 4, 16, 21, 25, 28, 29, 36, 38, 42), we find that autorepression by IE86 is dependent on the *crs* element in which direct binding of IE86 to this element (21, 25, 29) is a prerequisite for repression of transcription (reference 29 and Fig. 1). Transcription from eukaryotic protein-encoding genes is a sequential process involving preinitiation complex formation, initiation, elongation, and reinitiation steps which requires the orderly assembly and disassembly of transcriptional factors on the core promoter (50). In principle,

any one of these steps could be a point of control by promoter-selective factors such as IE86. Previous studies have suggested, but not proven, that the transcriptional repression mediated by IE86 probably involves a blockage of, or interaction with, the RNAPII transcription complex (21, 25, 29). In this report, we have demonstrated that IE86 directly inhibits the formation of transcription initiation complexes on the MIEP by specifically occluding the assembly of an RNAPII preinitiation complex. Evidence is also presented which indicates that IE86 is unable to block either reinitiation or elongation steps in the transcription process. Further, our experiments demonstrate that once a preinitiation complex is formed, IE86 is unable to effect inhibition.

The mode of IE86 repression revealed by this study is likely to have biological implications for understanding HCMV MIEP autoregulation during a permissive infection. First, these results indicate that, during an infection, the ability of IE86 to autorepress the MIEP will be dependent on the physical occupation of the promoter by transcription complexes. Thus, during the initial stages of an acute infection or during activation from a quiescent state upon cellular differentiation, the MIEP will be occupied by cellular transcription factors prior to the accumulation of IE86. At these stages of infection, IE86 would be predicted to be restricted in its ability to repress transcription from the MIEP. However, following DNA replication of the HCMV genome, the MIEP is likely to be stripped of transcription complexes, providing an ideal opportunity for IE86-mediated repression of the MIEP present in the newly replicated viral genomes. In this regard, it is important to note that during a permissive infection IE86 persists throughout the HCMV replication cycle (37, 41).

The second implication relates to the innate potency manifested by the enhancer domain of the HCMV MIEP. Strategically, if a strong promoter was required to be tightly autoregulated, a repressor would ideally be deployed to block an early step in the transcription process. The location of the IE86 binding site (*crs* element) in the vicinity of initiating complexes and the demonstrated ability of IE86 to inhibit preinitiation complex formation, one of the earliest steps in the transcription process, most likely reflect strong evolutionary pressure to regulate such a potent promoter. In addition to these possible implications, several important conclusions about the mechanism by which IE86 regulates transcription complex assembly follow from our results and are discussed below.

Possible mechanism by which IE86 protein negatively autoregulates transcription. While significant advances have been made in understanding the role of sequence-specific DNA-binding proteins in the selective activation of eukaryotic promoters, considerably less is known about the mechanism(s) by which regulatory proteins repress transcription. Several proposed models (which are not mutually exclusive) for transcriptional repression have been recently suggested (reviewed in reference 27). These include competition for an activator protein's DNA binding site, which is the simplest and probably the most common form of repression; quenching, in which the repressor interferes with the activation potential but not the binding of an activator protein; and direct repression, when the negative control factor directly blocks the activity of the basal transcription complex.

Our experiments support the notion that IE86 can function as a direct repressor, by interfering with the assembly of the basal transcription complex. Recently, a number of inhibitors of transcription complex assembly have been described (18, 31, 32). These inhibitory factors function by directly associating with TBP to block recruitment of preinitiation factors to promoter sites. Like these proteins, IE86 can interact with TBP, but our finding that IE86 derivatives that are unable to bind promoter-bound TBP can function as repressors indicates that IE86 inhibits basal transcription via a distinct mechanism. These findings are in agreement with a recent study by Macias and Stinski (29) in which a chimeric form of IE86 truncated at aa position 290 was also observed to repress MIEP transcription in vitro.

An alternative mechanism for direct repression of preinitiation complex assembly involves simply blocking the binding of general factors to the core promoter. Examples of this type of repression have been documented for both cellular and viral DNA-binding proteins (10, 23, 34). Similar to IE86, all of these proteins not only function as activator proteins but can also repress transcription from selective promoters (in a DNA-binding-dependent manner) by competing with the interaction of recombinant TBP (or native TFIID) with the TATA box (10, 23, 34). Previously, we have shown that the binding of IE86 to its cognate sequence element (*crs*) does not preclude recombinant TBP interactions with the TATA box (21), suggesting that the function of IE86 repression is different from these examples. However, it is plausible that IE86 will block the binding of the multisubunit native TFIID isolated from nuclei rather than that of the recombinant TBP subunit. Alternatively, repression mediated by IE86 binding to the *crs* element could involve the induction of a conformational change in the DNA that prevents the assembly of the preinitiation complex.

The results of this study, together with experimental evidence from other studies (21, 22, 25, 29), have eliminated a number of potential mechanisms and suggest that the IE86 protein can sterically interfere with the proper assembly of the preinitiation transcription complex. Since IE86 has been shown to bind to its DNA target (*crs*) prior to, or simultaneously with, TBP binding the TATA box, the binding of TFIIB (the next factor recruited to the preinitiation complex) is arguably the target of IE86-mediated repression. Moreover, TFIIB has previously been shown to interact in the DB complex distal to the TATA box in a position likely to be occupied by promoter-bound IE86 (50). Although this model is speculative, it offers an explanation for all the available data, as well as providing predictive value for future experiments.

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